*Am J Physiol Cell Physiol*
279: C1595–C1602, 2000.

# $Ca^{2+}$-ATPase protein expression in mammary tissue

TIMOTHY A. REINHARDT,[1] ADELAIDA G. FILOTEO,[2]
JOHN T. PENNISTON,[2] AND RONALD L. HORST[1]
[1]*Periparturient Diseases of Cattle Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, Iowa 50010; and* [2]*Department of Biochemistry and Molecular Biology, Mayo Foundation, Rochester, Minnesota 55905*

Received 24 April 2000; accepted in final form 26 June 2000

**Reinhardt, Timothy A., Adelaida G. Filoteo, John T. Penniston, and Ronald L. Horst.** $Ca^{2+}$-ATPase protein expression in mammary tissue. *Am J Physiol Cell Physiol* 279: C1595–C1602, 2000.—Protein expression of plasma membrane $Ca^{2+}$-ATPases (PMCAs) and the putative Golgi secretory pathway $Ca^{2+}$-ATPase (SPCA) was examined in rat mammary tissue. As lactation started, PMCA protein expression increased dramatically, and this increased expression paralleled milk production. Mammary PMCA was primarily PMCA2b but was ~4,000 daltons larger than expected. RT-PCR showed that the primary mammary PMCA2b transcript was alternatively spliced, at splice site A, to include an additional 135 bp, resulting in the insertion of 45 amino acids. This splice form is designated 2bw. PMCA2bw is secreted into milk, associated with the milk fat globule membrane. Therefore, PMCA2bw is located on the apical membrane of the secretory cell. Smaller amounts of PMCA1b and 4b protein were found in mammary tissue. PMCA4b was the major PMCA expressed in developing tissue, and its level declined as lactation started. PMCA1b expression increased moderately during lactation. SPCA protein expression increased 1 wk before parturition and increased further as lactation proceeded. The abundance and cell location of PMCA2b suggest that it is important for macro-$Ca^{2+}$ homeostasis in lactating tissue. The pattern of expression and abundance of SPCA suggest that it is a candidate for the Golgi $Ca^{2+}$-ATPase.

breast; mammary gland; calcium pumps; calcium-transporting ATPase

THE MAMMARY GLAND transports large amounts of $Ca^{2+}$ from the blood to milk via mammary secretory cells. These transcellular $Ca^{2+}$ fluxes, associated with lactation, must be rigorously regulated to prevent $Ca^{2+}$ cytotoxicity (29, 41, 44). This is a formidable task, because the mammary gland in mice and cows stores 12–30 μmol $Ca^{2+}$/g tissue compared with <1 μmol/g in nonmammary tissues (4, 39, 41). The large intracellular $Ca^{2+}$ pool in the mammary gland is thought to be concentrated in the Golgi apparatus (10), where millimolar $Ca^{2+}$ is essential for normal protein synthesis, processing, and secretion (8, 15). Therefore, a mechanism must be in place to move $Ca^{2+}$ into the Golgi.

Despite the importance of mammary cell $Ca^{2+}$ homeostasis, little is known about the proteins involved in $Ca^{2+}$ transport and their regulation (44). Biochemical evidence exists for a P-type $Ca^{2+}$-ATPase in the Golgi of mammary tissue with characteristics slightly different from either the plasma membrane $Ca^{2+}$-ATPase (PMCA) or the sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase (SERCA) (4, 5, 15, 40, 50). This mammary Golgi secretory pathway $Ca^{2+}$-ATPase (SPCA) could be a homologue to the yeast Golgi $Ca^{2+}$-ATPase PMR1 (16, 45), such as the putative rat SPCA (26). It has also been suggested that there is no unique mammalian Golgi $Ca^{2+}$-ATPase but, rather, that SERCAs in the endoplasmic reticulum (ER) and PMCAs in transit to the plasma membrane maintain the $Ca^{2+}$ concentrations found in the Golgi (49). Previous work has shown that the putative rat SPCA mRNA is expressed in the mammary gland and is associated with milk production (44). Furthermore, the pattern of SPCA expression is consistent with the biochemical evidence for a Golgi SPCA in lactating tissue.

In addition to being pumped into the Golgi, $Ca^{2+}$ must be effectively removed from the mammary cell and pumped into the milk. PMCAs represent a ubiquitous, high-affinity system for removal of $Ca^{2+}$ from the cell. PMCAs, along with SERCAs, are responsible for the long-term setting and maintenance of intracellular $Ca^{2+}$ levels (1, 11–13, 23, 28). Mammalian PMCAs are encoded by a multigene family consisting of four members termed PMCAs 1–4 (7, 11, 23). Cloning has revealed some 20–30 isoforms of the pump, generated by the alternative splicing of the primary gene transcripts at two sites identified as A and C (2, 20, 27, 28, 34, 38, 48). Alternative splicing of site C (COOH-terminal tail) has been shown to alter the regulatory properties of PMCA isoforms, particularly with respect to phosphorylation and calmodulin stimulation (17, 18, 23). Alternate splicing at site A has been shown to occur in PMCA2 (2, 27). No specific function has yet been found for this splice site (28), and only the z splice form of the protein has been demonstrated (20). The distribution of PMCA mRNA in rat and human tissues reveals that gene products 1 and 4 are ubiquitous, whereas gene

Address for reprint requests and other correspondence: T. A. Reinhardt, Periparturient Diseases of Cattle Research Unit, National Animal Disease Center, Agricultural Research Service, USDA, Ames, IA 50010 (E-mail: treinhar@nadc.ars.usda.gov).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

products 2 and 3 are tissue specific and are found enriched in nervous tissue (22). Early protein studies have confirmed this pattern of expression for the four isoforms. It has also been shown that the splice variants of each isoform are expressed in a tissue- and cell type-specific manner (20, 23, 46–48).

Previous work has shown that PMCA isoforms 1b, 2b, and 4b, but not 3, are expressed in rat mammary tissue (44). PMCA2b mRNA was the most abundant PMCA mRNA expressed, and its expression was high in the lactating mammary gland of the rat (44). These data suggested that PMCA2b mRNA expression in the mammary gland is highly regulated by a factor(s) controlling milk production. This is the first evidence of significant expression of the PMCA2 isoform in a tissue other than brain. Thus it is suggested that PMCA2b might play a key role by regulating intracellular $Ca^{2+}$ concentrations where large transcellular $Ca^{2+}$ fluxes are required for milk production.

In this paper we present evidence for high expression of SPCA and PMCA2b protein in rat lactating mammary tissue. Interestingly, we have evidence that alternate splicing at site A in the mammary gland is different than that observed in the brain, where the shorter z splice form of PMCA2b is predominant (2, 20). We will also demonstrate that the PMCA2bw transcript and the correspondingly larger 2bw protein are the primary PMCA forms observed in lactating mammary tissue, whereas PMCA4b is the primary PMCA protein in developing mammary tissue.

## MATERIALS AND METHODS

*Animals.* The National Animal Disease Center's Animal Care and Use Committee approved all animal procedures. Confirmed-pregnant Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Madison, WI). Rats were housed, individually, in hanging basket cages with sawdust bedding. Starting 11 days after the breeding date, rats were killed at 10, 7, and 1 day prepartum and 1, 7, 14, 18, and 21 days postpartum. Data are presented as days, with parturition as the reference point: negative days indicate days before parturition, and positive days indicate days of lactation (i.e., after parturition). Rats were anesthetized with a 50:50 mix of $C0_2$-$O_2$ and then decapitated. Rats were confirmed pregnant by inspection of the uterus. Mammary tissue and other tissues were removed, flash frozen in liquid $N_2$, and stored at −70°C until RNA or membranes were prepared.

*Tissue and milk fat globule membrane preparation.* Tissue microsomes were prepared as previously described (14). Briefly, tissue was homogenized in 10 vol of *buffer A*, containing 10 mM Tris·HCl, 2 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 4 μg/ml aprotinin, and 4 μg/ml leupeptin at pH 7.5. An aliquot of the homogenate was saved for DNA analysis (9). The homogenate was mixed with an equal volume of *buffer B* (*buffer A* containing 0.3 M KCl) and centrifuged at 4,000 *g* for 10 min. The supernatant was collected, adjusted to 0.7 M KCl by the addition of solid KCl, and centrifuged at 100,000 *g* for 1 h. The supernatant was discarded, and the pellets were resuspended in *buffer C* (*buffer A* containing 0.15 M KCl). Membrane preparations were stored at −70°C until assayed.

Milk was collected from rats on *days 14–18* of lactation. The milk was cooled to 4°C and centrifuged at 10,000 *g* for 15 min. The floating milk fat pellet was removed, mixed with 5 vol of PBS plus complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN), and centrifuged at 10,000 *g* for 15 min. This step was repeated one to two times or until the supernatant was clear. The washed fat pellet was processed in the same manner as tissue from this point forward to yield milk fat globule membranes (MFGMs). Proteins were determined with the Bio-Rad Protein Assay Kit using a BSA standard.

*Gel electrophoresis and Western blotting.* The methods were basically as described previously (20). Briefly, microsomes were incubated for 15 min at room temperature in a modified Laemmli buffer containing 150 mg/ml urea and 65 mM dithiothreitol. Samples were then electrophoresed for 1.5 h at 125 V in a 6% Tris-glycine gel (Novex, San Diego, CA). Proteins were transferred to nitrocellulose membranes for 1 h at 25 V in 0.7 M glycine-0.025 M Tris at pH 7.4. Blots were developed with Supersignal (Pierce, Rockford, IL) by using the protocol provided by the manufacturer. Anti-PMCA antibodies 5F10, NR1, NR2, and NR4, described previously (20), were used at 1:20,000, 1:1,000, 1:1,000, and 1:5,000 dilutions, respectively. Antibody 220 (final dilution 1:5,000) is a PMCA2-specific antibody similar to the previously described NR2 (20). Antibody 220 was prepared by using the same peptide sequence (TNSDFYSKNQRNESSC) used to make NR2. The peptide sequence used to make the SPCA antibody (final dilution 1:5,000) was VARFQKIPNVENETMIC (26). Rabbits were immunized with peptide, which was synthesized by Multiple Peptide Systems (San Diego, CA) and conjugated to keyhole limpet hemocyanin. The general procedures were described previously (35). Antibodies 220 (anti-PMCA2) and anti-SPCA were characterized as follows. Antibody 220 gave the same results as the previously characterized NR2, and preincubation of antibody 220 with the PMCA2 peptide TNSDFYSKNQRNESSC eliminated specific banding on Western blots. Preincubation of antibody 220 with an irrelevant peptide yielded normal PMCA2 blots. Preincubation of SPCA antibody with the SPCA peptide VARFQKIPNVENETMIC eliminated specific banding on Western blots. Preincubation of the SPCA antibody with an irrelevant peptide yielded normal SPCA blots with bands of the predicted size.

*RNA preparation and Northern blot analysis.* Total RNA and DNA were isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction using Trizol reagent (GIBCO-BRL, Gaithersburg, MD). Poly(A)$^+$ RNA was prepared using the Poly A Tract mRNA Isolation System IV (Promega, Madison, WI). Poly(A)$^+$ RNA (2–3 μg) was fractionated in formaldehyde-agarose gels, and blotting and hybridization were performed as previously described (31). PMCA2 and SPCA probes were prepared by RT-PCR as described previously. After hybridization, the blots were imaged and quantitated on an InstantImager (Packard Instruments, Downers Grove, IL).

*RT-PCR and sequencing.* The primers used for RT-PCR of splice site A in PMCA2b were 5′-TGAAAGCTCGCTCACAGGG-3′ and 5′-TCAGAGGCTGCATTTCCATAG-3′, as described previously (2). PCR was carried out in a GeneAmp 9600 PCR System (Perkin Elmer, Foster City, CA). The RT-PCR reaction master mix (99 μl) consisted of 10 μl of 10× reaction buffer (Boehringer Mannheim), 77.75 μl of diethyl pyrocarbonate-treated water, 8 μl of dNTPs of 10 mM stock (Amresco, Solon, OH), 1 μl at 100 pmol/μl of each of the specific primers (IDT, Coralville, IA), 0.25 μl (50 units) MMLV (GIBCO-BRL), 0.5 μl (20 units) RNAsin (Promega), and 0.5 μl (2.5 units) *Taq* polymerase (GIBCO-BRL). To this master mix we added 1 μl of template (1 μg RNA). The RT

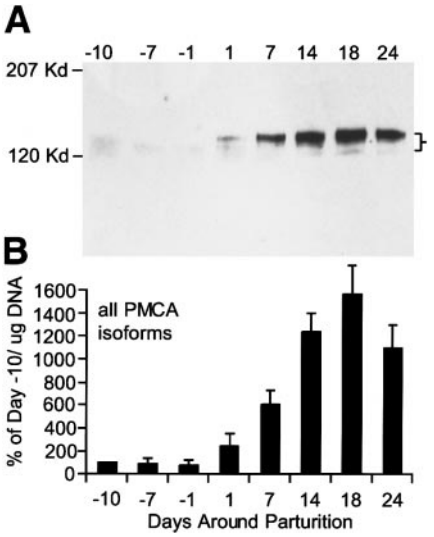



Fig. 1. *A*: general plasma membrane $Ca^{2+}$-ATPase (PMCA) protein expression in rat mammary tissue during pregnancy and lactation, as determined by Western blot with the use of an antibody (5F10) that recognizes all PMCA isoforms. *B*: normalized expression pattern changes expressed as a percentage of expression relative to that on *day −10* (per μg tissue DNA). Data are means ± SE ($n$ = 3–5 animals tested on 3–5 gels) presented in days, with parturition as the reference point: negative days indicate days before parturition, and positive days indicate days of lactation (i.e., after parturition).

reaction was incubated for 40 min at 56°C, followed by 4 min at 95°C. PCR was 25 cycles at 94°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The run was completed with a 20-min extension step at 72°C. PCR products of the expected band size were sampled and reamplified using the same primers. The reamplified products were purified and submitted to the Iowa State University Sequencing Facility for direct PCR sequencing.

*Statistical analysis.* Differences in pump expression, as affected by day, were analyzed by least-squares ANOVA. When day effects were significant, specific comparisons were made using Tukey's test. All analysis was performed using StatView (SAS Institute, Cary, NC).

## RESULTS

*PMCA expression in rat mammary tissue as affected by pregnancy and lactation.* The patterns of $Ca^{2+}$-ATPase protein expression in mammary tissue of rats 11 days pregnant to 21 days lactating were examined by Western blot. Figure 1*A* shows a typical blot representing the expression of total PMCA. Using an antibody (5F10) that recognizes all PMCA isoforms, we found small amounts of PMCA protein in the mammary gland before parturition. It was not until lactation started that total PMCA protein increased significantly ($P < 0.1$). PMCA protein expression increased 15 times by *day 18* of lactation ($P < 0.001$) compared with that at *day 11* of pregnancy (Fig. 1). This increase in total PMCA paralleled milk production. Using isoform-specific antibodies (Fig. 2), we found that PMCA1b protein was present at low levels on day −10. PMCA1b protein doubled in late pregnancy and almost doubled again by peak lactation on *day 18* ($P < 0.01$) (Fig. 2*A*). As shown in Fig. 1, the developing mammary gland of pregnant rats expressed relatively low amounts of PMCA compared with the levels expressed in the lactating mammary gland. Figure 2*B* also shows that this relatively small amount of PMCA in developing mammary tissue is primarily PMCA4b. PMCA4b expression increased 2.5 times ($P < 0.05$) from *day 11* of pregnancy to *day 1* of lactation. As lactation increased, PMCA4b expression in the mammary gland declined significantly ($P < 0.001$). Since PMCA1b (Fig. 2*A*) increased only moderately during lactation, while PMCA4b was rapidly declining in lactation (Fig. 2*C*), the large increase in PMCA cannot be attributed to isoforms 1 and 4. Probing the blots with anti-PMCA2 antibodies showed that the large increase in PMCA protein expression is due primarily to PMCA2b (Fig. 2*B*). PMCA2b protein expression increased significantly on *day 1* of lactation ($P < 0.001$) and increased almost 100 times by *day 18* of lactation ($P < 0.001$) compared with that at *day 11* of pregnancy.

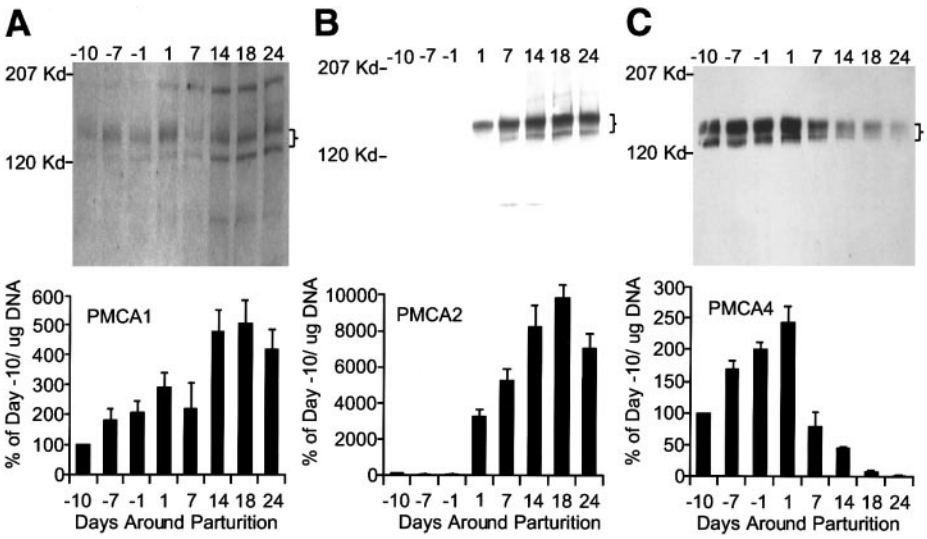



Fig. 2. *Top*: PMCA1, 2, and 4 protein expression in rat mammary tissue during pregnancy and lactation, as determined by Western blot using antibody NR1, which recognizes PMCA1 (*A*), antibody 220, which recognizes PMCA2 (*B*), and antibody JA9, which recognizes PMCA4 (*C*). *Bottom*: normalized expression pattern changes expressed as a percentage of expression relative to that on *day −10*. Data are means ± SE ($n$ = 3–5 animals tested on 3–5 gels) presented in days.

*Evidence for alternate splicing of PMCA2b, at splice site A, in mammary tissue.* Western blotting showed that mammary PMCA2b was ~4,000 daltons larger than expected compared with brain PMCA2b (Fig. 3*A*) or recombinant PMCA2bz (data not shown). RT-PCR showed that the primary mammary PMCA2b transcript was alternatively spliced, at splice site A, to include an additional 135 bp resulting in the insertion of 45 amino acids (Fig. 3*B*). This splice form is designated 2bw and accounts for the 4,000-dalton increase observed for mammary PMCA2b. The identity of the PCR products in Fig. 3 was confirmed by size and sequencing. RT-PCR showed that the w splice form was the predominant PMCA2 transcript in the mammary gland, kidney, uterus, and liver (Fig. 3*B*). However, only the mammary gland expressed PMCA2bw protein in abundance, with small amounts of PMCA2bw seen in the liver (Fig. 3*A*).

Figure 4 shows a comparison of transcript and protein expression of PMCA2 in mammary gland and brain. Mammary gland expresses four times more PMCA2b mRNA per microgram of DNA than the brain, but the brain expresses three to four times more PMCA2b protein. The other tissues tested expressed very small amounts of PMCA2b transcript and protein per microgram of DNA. The finding of greater PMCA2b mRNA in the mammary gland, but less protein than in the brain, suggests a significantly shorter half-life for PMCA2b in the mammary gland compared with the brain.

*Putative (Golgi) SPCA expression in rat mammary tissue as affected by pregnancy and lactation.* Using an antibody specific for the putative rat SPCA, we found that SPCA protein expression was evident in the mammary gland at *day 11* (*day −10*) of pregnancy (Fig. 5). SPCA protein expression increased three times ($P < 0.01$) by *day 14* (*day −7*) of pregnancy. SPCA protein increased further at the start of lactation and remained elevated ($P < 0.001$) at six to seven times the level seen at *day 11* of pregnancy through *day 18* of lactation (Fig. 5, *A* and *B*). Western blotting showed that a form of mammary SPCA was ~2,000 daltons larger than expected when compared with brain or testes SPCA (Fig. 5*C*). RT-PCR, with primers covering most of the open reading frame (bases 205–2,858) of rat SPCA (GenBank accession no. M93017), failed to find evidence for an alternatively spliced form of SPCA. This suggests that alternate splicing occurs outside the region examined or that the increased size of mammary SPCA is due to posttranslational modifications. Preincubation of the SPCA antibody with the SPCA peptide used to prepare the antibody eliminated specific banding on the Western blots (data not shown). The data suggest a mammary-specific SPCA splice form because the mammary form was not found in any other tissues tested. The splice must occur at the extreme amino or carboxyl terminus.

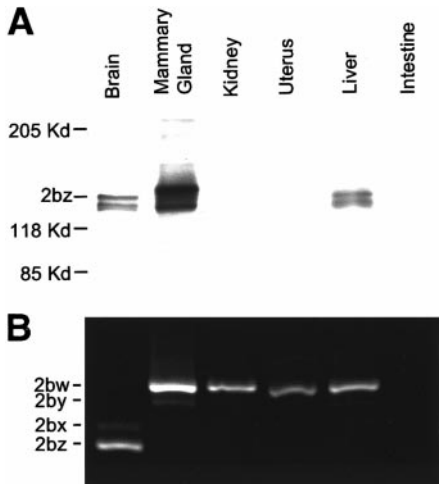



Fig. 3. Evidence for alternative splicing of PMCA2 in mammary tissue as determined by Western blotting (*A*) and RT-PCR (*B*). *A*: Western blot performed with a PMCA2-specific antibody. The amount of membrane protein loaded was 1 μg of brain, 10 μg of mammary tissue, and 40 μg for each of the remaining tissues. *B*: RT-PCR of rat brain, lactating mammary tissue, kidney, uterus, liver, and intestine RNA using primers that flank splice site A in PMCA2. Presence of alternatively spliced transcripts 2bz, 2bx, 2by, and 2bw was confirmed by product size (242, 284, 335, and 377 bp, respectively) and sequencing.

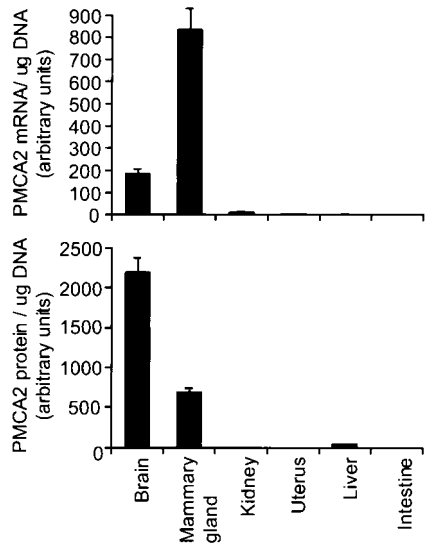



Fig. 4. Relative expression of PMCA 2b mRNA (*top*) and protein (*bottom*) in brain, lactating mammary tissue, kidney, uterus, liver, and intestine.

Figure 6 shows that the mammary gland expressed seven to eight times more SPCA mRNA per microgram of DNA than did the brain but only two to three times more SPCA protein than the brain. While this difference is not as dramatic as that seen for PMCA2 (Fig. 4), it does suggest that SPCA protein is turning over slightly faster in the mammary gland than in the brain.

*Secretion of $Ca^{2+}$-ATPases into milk on the MFGMs.* The fat in milk is a lipid droplet surrounded by a membrane. The source of this membrane is the apical membrane of the milk secretory cell. Therefore, examination of MFGMs yields information about the proteins located on the apical membrane of the mammary secretory cell. Using antibody 5F10, which recognizes

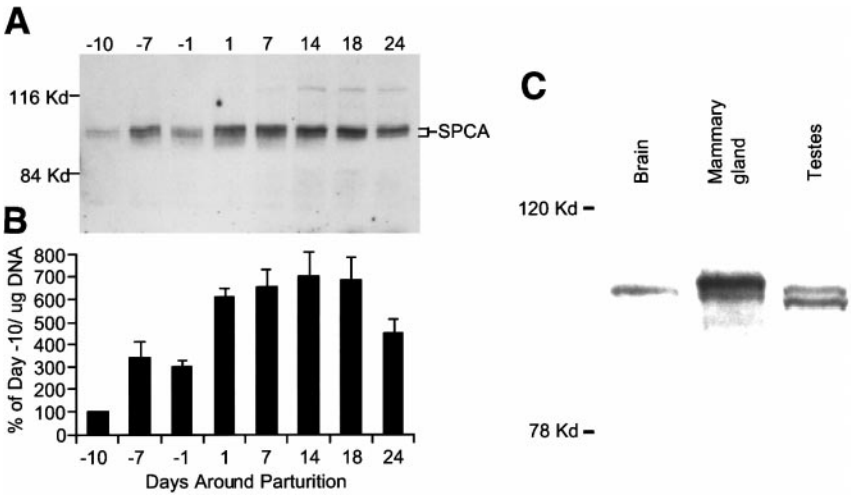



Fig. 5. *A*: secretory pathway $Ca^{2+}$-ATPase (SPCA) protein expression in rat mammary tissue during pregnancy and lactation, as determined by Western blotting. *B*: changes in SPCA expression relative to that on *day −10*. Data are means ± SE ($n$ = 3–5 animals tested on 3–5 gels) presented in days. *C*: a Western blot of brain, mammary gland, and testes performed with an SPCA-specific antibody. The amount of membrane protein loaded was 8 μg of brain, 15 μg of mammary tissue, and 25 μg of testes membrane protein.

all PMCA isoforms, we showed that significant amounts of PMCAs were associated and secreted into milk with the MFGM (Fig. 7, *top left*). PMCAs in MFGMs were enriched eight times (Fig. 7, *bottom*) more than PMCA measured in mammary tissue membranes (see Fig. 7 legend for protein loading differences between blots and membrane sources). Anti-PMCA2 antibody showed that PMCA2 was 21 times more concentrated in MFGMs than in mammary tissue membranes. Small amounts of PMCA1b and 4b were associated with the MFGM (data not shown), but PMCA2 accounted for the majority of PMCA in MFGMs. In contrast to the enrichment of PMCAs in MFGMs, SPCA (Fig. 7, *top right*) was found to be primarily associated with mammary membranes with very little SPCA found associated with MFGMs.

*Comparison of $Ca^{2+}$-ATPases expression in mammary tissue to other tissues.* Figure 8 shows the relative expression of PMCAs, PMCA2, PMCA4, and SPCA in mammary tissue compared with that in the brain, kidney, uterus, liver, and intestine (note different amounts on membranes loaded by tissue type). Except for the brain, the lactating mammary gland expresses more $Ca^{2+}$-ATPases than most tissues.

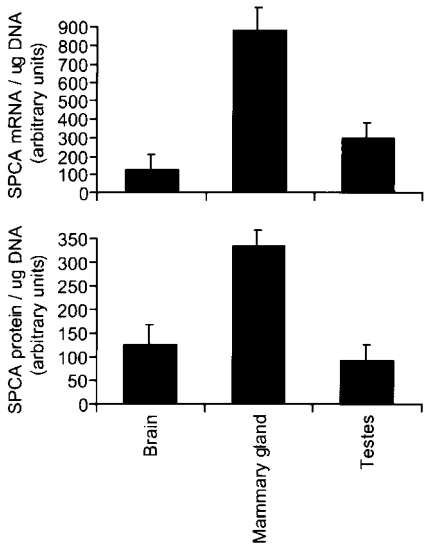
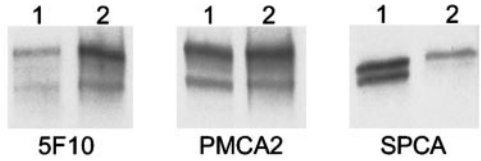



Fig. 6. Relative expression of SPCA mRNA (*top*) and protein (*bottom*) in brain, lactating mammary tissue, and testes.

## DISCUSSION

The lactating mammary gland is the site of significant $Ca^{2+}$ storage and transport, but despite the importance of mammary cell $Ca^{2+}$ homeostasis, little is known about the proteins that regulate it or their expression (29, 41). Previously, we identified the $Ca^{2+}$-ATPases (PMCA1b, 2b, 4b, and SPCA) present in mammary tissue and examined their mRNA expres-




| Antibody used | Mammary membranes | Milk fat globule membranes | Fold enrichment over mammary membranes |
|---|---|---|---|
| | arbitrary units/ ug protein | | |
| 5F10 | 66 | 570 | 8.6 |
| PMCA2 | 33 | 720 | 21.8 |
| SPCA | 341 | 40 | -8.5 |

Fig. 7. Western blots comparing mammary gland microsomes (*lane 1*) and milk fat globule membranes (*lane 2*) isolated from rats on *day 14* of lactation. For the blots performed with the general PMCA antibody (5F10) and the anti-SPCA antibody, *lane 1* was loaded with 15 μg of mammary membrane protein and *lane 2* was loaded with 25 μg of milk fat globule membrane protein. The anti-PMCA2 blot had 30 μg of mammary membrane protein in *lane 1* and 5 μg of milk fat globule membranes in *lane 2*. The table shows the relative abundance of pump types in each membrane preparation and the enrichment, if any, in the milk fat globule membranes.

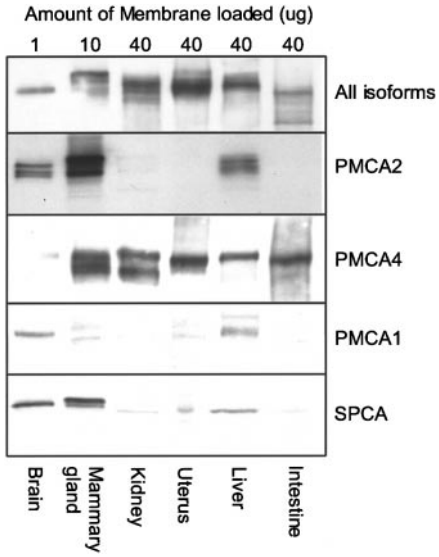



Fig. 8. Western blots of rat brain, lactating mammary tissue, kidney, uterus, liver, and intestinal membranes performed with the antibodies indicated (*right*). Note that different tissues were loaded with different amounts of protein.

sion during development and lactation (44). We concluded that (on the basis of $Ca^{2+}$ pump mRNA expression) the comparatively low expression of PMCA1b and 4b limited the potential role of these two $Ca^{2+}$-ATPases in macro-$Ca^{2+}$ transport in the mammary gland. The high level of expression of PMCA2b and the putative SPCA mRNAs, as well as the timing of their expression relative to $Ca^{2+}$ transport and storage, suggested that these $Ca^{2+}$ transporters are key regulators of macro-$Ca^{2+}$ transport in the mammary gland. These studies did not address whether the mRNA expression data was reflected in protein expression for the $Ca^{2+}$-ATPases. The present study shows that our previous mRNA data are generally correlated with pump protein expression and provide new information about PMCA2 splice forms and their cellular location in mammary tissue. The exception is PMCA4b. PMCA4 mRNA was increased in lactating tissue, but PMCA4 protein declined significantly during lactation.

Using 5F10, an antibody that binds all PMCAs equally, we found that PMCA protein expression increased significantly and paralleled milk production (Fig. 1). This increased PMCA expression in mammary tissue was primarily PMCA2b (Fig. 2), because PMCA1b increased only slightly and PMCA4b declined significantly as lactation increased. The observed downregulation of PMCA4b, while PMCA2b was developmentally upregulated, in lactating mammary tissue resembles the regulation of these PMCA2 and 4 isoforms in cultured cerebellar granule cells as they develop (24, 25). The downregulation of PMCA4 and upregulation of PMCA2 in granule cells is associated with increased influxes of $Ca^{2+}$, which are constantly occurring in lactating mammary tissue. Peak lactation in mammary tissue resulted in higher total PMCA despite the marked downregulation of PMCA4. Furthermore, the major portion of total mammary pump protein was PMCA2, similar to findings in cultured granule cells at peak development.

The significant expression of PMCA2 transcripts and protein in lactating mammary tissue (Figs. 2 and 3) is contrary to the popular belief that PMCA2 is only present in significant amounts in brain (23). Figure 4 shows that PMCA2 mRNA in the mammary gland is four times that observed in brain, whereas PMCA2 protein levels are one-third that seen in brain. The higher mRNA but lower PMCA2 protein seen in mammary tissue vs. brain suggests a much shorter half-life for mammary PMCA2 than the reported 12-day half-life for brain PMCAs (19). A significantly shorter half-life for PMCA2 protein in the mammary gland is supported by the finding that PMCA2 is concentrated and secreted into the milk on the MFGM (Fig. 7). The source of MFGM is the apical membrane of the mammary secretory cell. This apical membrane surrounds milk fat as it is secreted into milk, and it is estimated that the apical membrane is completely lost and replaced three times a day as a part of the process of milk fat secretion (21, 32, 33). Consequently, the high expression of PMCA2 mRNA in the mammary gland is explained by the need to rapidly replace PMCA2 lost to milk secretion. The concentration of PMCA2 in the MFMG suggests that PMCA2 is primarily located on the apical membrane. The mammary secretory cell transports $Ca^{2+}$ from the basolateral to the apical side of the cell. The apical location of most of the mammary PMCA contrasts with that in the kidney and intestine, where PMCAs are localized in the basolateral membrane and are also involved in macrotranscellular $Ca^{2+}$ fluxes (6, 51). This raises the question as to how PMCAs might be selectively targeted to apical or basolateral membranes in polarized epithelial cells.

Isoform-specific antibodies showed that mammary PMCA was primarily PMCA2b but that it was ~4,000 daltons larger than expected. RT-PCR showed that the primary mammary PMCA2b transcript was alternatively spliced, at splice site A, to include an additional 135 bp resulting in the insertion of 45 amino acids. This splice form is designated 2bw (2) and accounts for the 4,000-dalton increase in size of mammary PMCA2b. Only the mammary gland expressed PMCA2bw protein in abundance, with small amounts of PMCA2bw found in the liver (Fig. 3). The significance of this splice form remains to be determined because no functional differences resulting from alternate splicing at site A have been detected with the methods used (28). Although its role is yet unknown, the w splice form could play a role in targeting PMCA2 to the apical membrane of the mammary secretory cell.

The putative Golgi $Ca^{2+}$-ATPase, SPCA, is the only protein that increased significantly before parturition (Fig. 5). This finding is similar to results in our previous study in which the mRNA for SPCA was the only transcript to increase before parturition (44). This is significant because this is the time when the animal becomes hypocalcemic due to rapid influx of $Ca^{2+}$ to mammary stores. Clear biochemical evidence exists for a Golgi $Ca^{2+}$-ATPase important to mammary function

in general and casein micelles synthesis in particular (4, 5, 8, 15, 39, 40, 50). In yeast, the PMR1 gene product is a $Ca^{2+}$-ATPase located in the Golgi complex of yeast (3, 16, 42, 45). Rat, human, and bovine homologues of the yeast PMR1 have been detected by cloning, but their subcellular location and specific function remain to be determined (26, 30). Mutations in the human PMR1 homologue have been shown to be the cause of Hailey-Hailey disease, in which cultured keratinocytes from these patients have impaired regulation of cytoplasmic $Ca^{2+}$ (30). Together, the data for yeast and humans suggest that the mammalian PMR1 homologue could be a mammalian Golgi $Ca^{2+}$-ATPase. The opposing argument put forth by Taylor et al. (49) is that the Golgi complex does not contain a unique "resident" $Ca^{2+}$-ATPase. They state that all $Ca^{2+}$ uptake into Golgi can be attributed to PMCAs in transit to the plasma membrane and to SERCAs that are not restricted to the ER. The presence of a unique resident $Ca^{2+}$-ATPase in the Golgi remains to be determined, but data presented here and elsewhere suggest that SPCA is a candidate (26, 30, 44, 45).

Whether SPCA is determined to be a Golgi $Ca2^{+}$-ATPase or not, transport and retention of calcium by the Golgi is important to mammary cell function (8, 15) and to cell function in general (36, 37, 43). As noted above, the high expression of PMCA2 mRNA in the mammary gland is explained by the need to rapidly replace PMCA2 protein lost to milk secretion. This need to rapidly replace PMCA2 lost to the rapid turnover of the apical membrane of the mammary secretory cell also should result in significant PMCA2 in transit in the Golgi. It is reasonable, on the basis of the findings of Taylor et al. (49), that the large amount of PMCA2 in transit to the apical membrane may make a significant contribution to Golgi $Ca^{2+}$ in the mammary gland. The contribution of PMCA2s to Golgi $Ca^{2+}$ would appear to be restricted to after the start of lactation, because there is little PMCA2 present before significant milk production (Fig. 2). The mammary gland needs to accumulate $Ca^{2+}$ before the initiation of lactation. The significant expression of SPCA before the start of parturition and lactation is therefore significant, because this putative $Ca^{2+}$-ATPase is the only $Ca^{2+}$-ATPase to increase significantly before parturition.

In summary, this study demonstrates that the initiation of lactation and increasing milk production are associated with significant PMCA expression in the mammary gland. The primary PMCA expressed is the unique isoform PMCA2bw. PMCA2bw is apparently synthesized at a high rate to maintain its concentration in the apical membrane of the secretory cell, where it is rapidly lost to milk secretion. The abundance, cell location, high affinity for $Ca^{2+}$, and high constitutive activity of PMCA2bw suggest that PMCA2b is important for macro-$Ca^{2+}$ homeostasis in lactating tissue. The putative Golgi $Ca^{2+}$-ATPase, SPCA, is the only $Ca^{2+}$-ATPase whose expression increases significantly before parturition at a time of significant $Ca^{2+}$ accumulation by the mammary gland. The pattern of expression and the abundance of SPCA suggest that it is a candidate for the Golgi $Ca^{2+}$-ATPase shown to be important in maintaining the Golgi $Ca^{2+}$ concentration required for casein synthesis. Several questions remain to be addressed, specifically with regard to the nature and function of SPCA and whether the large amount of PMCA2bw being made contributes to Golgi $Ca^{2+}$ as it transits to the plasma membrane.

We thank Becky Baccam and Derrel Hoy for excellent technical assistance and Annette Bates for expert preparation of the manuscript.

## REFERENCES


1. **Abramowitz J and Suki WN.** Ca-ATPase and bone cell mineralization. *Miner Electrolyte Metab* 22: 336–344, 1996.
2. **Adamo HP and Penniston JT.** New $Ca^{2+}$ pump isoforms generated by alternative splicing of rPMCA2 mRNA. *Biochem J* 283: 355–359, 1992.
3. **Antebi A and Fink GR.** The yeast Ca(2+)-ATPase homologue, PMR1, is required for normal Golgi function and localizes in a novel Golgi-like distribution. *Mol Biol Cell* 3: 633–654, 1992.
4. **Baumrucker CR.** Calcium transport in lactation. In: *Lactation: A Comprehensive Treatise*, edited by Larson BL. New York: Academic, 1978, p. 463–474.
5. **Bingham EW, McGranaghan MB, Wickham ED, Leung CT, and Farrell HM Jr.** Properties of [$Ca^{2+}$ + $Mg^{2+}$]-adenosine triphosphatases in the Golgi apparatus and microsomes of the lactating mammary glands of cows. *J Dairy Sci* 76: 393–400, 1993.
6. **Borke JL, Zaki A e-M, Eisenmann DR, and Mednieks MI.** Localization of plasma membrane $Ca^{2+}$ pump mRNA and protein in human ameloblasts by in situ hybridization and immunohistochemistry. *Connect Tissue Res* 33: 139–144, 1995.
7. **Brandt PC and Vanaman TC.** The plasma membrane calcium pump: not just another pretty ion translocase. *Glycobiology* 6: 665–668, 1996.
8. **Burgoyne RD, Duncan JS, and Sudlow AW.** Role of calcium in the pathway for milk protein secretion and possible relevance for mammary gland physiology. *Biochem Soc Symp* 63: 91–100, 1998.
9. **Burton K.** A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemistry* 62: 315–323, 1955.
10. **Cameron IL, Sparks RL, and Seelig LL Jr.** Concentration of calcium and other elements at a subcellular level in the lactating epithelium of rat. *Cytobios* 27: 89–96, 1980.
11. **Carafoli E.** Plasma membrane calcium pump: structure, function and relationships. *Basic Res Cardiol* 92: 59–61, 1997.
12. **Carafoli E and Chiesi M.** Calcium pumps in the plasma and intracellular membranes. *Curr Top Cell Regul* 32: 209–241, 1992.
13. **Carafoli E, Garcia-Martin E, and Guerini D.** The plasma membrane calcium pump: recent developments and future perspectives. *Experientia* 52: 1091–1100, 1996.
14. **Caride AJ, Filoteo AG, Enyedi A, Verma AK, and Penniston JT.** Detection of isoform 4 of the plasma membrane calcium pump in human tissues by using isoform-specific monoclonal antibodies. *Biochem J* 316: 353–359, 1996.
15. **Duncan JS and Burgoyne RD.** Characterization of the effects of $Ca^{2+}$ depletion on the synthesis, phosphorylation and secretion of caseins in lactating mammary epithelial cells. *Biochem J* 317: 487–493, 1996.
16. **Durr G, Strayle J, Plemper R, Elbs S, Klee SK, Catty P, Wolf DH, and Rudolph HK.** The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with $Ca^{2+}$ and $Mn^{2+}$ required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. *Mol Biol Cell* 9: 1149–1162, 1998.
17. **Elwess NL, Filoteo AG, Enyedi A, and Penniston JT.** Plasma membrane $Ca^{2+}$ pump isoforms 2a and 2b are unusually

responsive to calmodulin and $Ca^{2+}$. *J Biol Chem* 272: 17981–17986, 1997.
18. **Enyedi A, Verma AK, Heim R, Adamo HP, Filoteo AG, Strehler EE, and Penniston JT.** The $Ca^{2+}$ affinity of the plasma membrane $Ca^{2+}$ pump is controlled by alternative splicing. *J Biol Chem* 269: 41–43, 1994.
19. **Ferrington DA, Chen X, Krainev AG, Michaelis EK, and Bigelow DJ.** Protein half-lives of calmodulin and the plasma membrane Ca-ATPase in rat brain. *Biochem Biophys Res Commun* 237: 163–165, 1997.
20. **Filoteo AG, Elwess NL, Enyedi A, Caride A, Aung HH, and Penniston JT.** Plasma membrane $Ca^{2+}$ pump in rat brain. Patterns of alternative splices seen by isoform-specific antibodies. *J Biol Chem* 272: 23741–23747, 1997.
21. **Franke WW, Luder MR, Kartenbeck J, Zerban H, and Keenan TW.** Involvement of vesicle coat material in casein secretion and surface regeneration. *J Cell Biol* 69: 173–195, 1976.
22. **Guerini D.** The significance of the isoforms of plasma membrane calcium ATPase. *Cell Tissue Res* 292: 191–197, 1998.
23. **Guerini D and Carafoli E.** The calcium pumps. In: *Calcium As A Cellular Regulator*, edited by Carafoli E and Klee CB. New York: Oxford Univ. Press, 1999, p. 249–278.
24. **Guerini D, Garcia-Martin E, Gerber A, Volbracht C, Leist M, Merino CG, and Carafoli E.** The expression of plasma membrane $Ca^{2+}$ pump isoforms in cerebellar granule neurons is modulated by $Ca^{2+}$. *J Biol Chem* 274: 1667–1676, 1999.
25. **Guerini D, Wang X, Li L, Genazzani A, and Carafoli E.** Calcineurin controls the expression of isoform 4CII of the plasma membrane Ca(2+) pump in neurons. *J Biol Chem* 275: 3706–3712, 2000.
26. **Gunteski-Hamblin AM, Clarke DM, and Shull GE.** Molecular cloning and tissue distribution of alternatively spliced mRNAs encoding possible mammalian homologues of the yeast secretory pathway calcium pump. *Biochemistry* 31: 7600–7608, 1992.
27. **Heim R, Hug M, Iwata T, Strehler EE, and Carafoli E.** Microdiversity of human-plasma-membrane calcium-pump isoform 2 generated by alternative RNA splicing in the N-terminal coding region. *Eur J Biochem* 205: 333–340, 1992.
28. **Hilfiker H, Guerini D, and Carafoli E.** Cloning and expression of isoform 2 of the human plasma membrane $Ca^{2+}$ ATPase. Functional properties of the enzyme and its splicing products. *J Biol Chem* 269: 26178–26183, 1994.
29. **Horst RL, Goff JP, and Reinhardt TA.** Calcium and vitamin D metabolism during lactation. *J Mammary Gland Biol Neoplasia* 2: 253–263, 1997.
30. **Hu Z, Bonifas JM, Beech J, Bench G, Shigihara T, Ogawa H, Ikeda S, Mauro T, and Epstein EH Jr.** Mutations in ATP2C1, encoding a calcium pump, cause Hailey-Hailey disease. *Nat Genet* 24: 61–65, 2000.
31. **Johnson JA, Beckman MJ, Pansini-Porta A, Christakos S, Bruns ME, Beitz DC, Horst RL, and Reinhardt TA.** Age and gender effects on 1,25-dihydroxy-vitamin $D_3$-regulated gene expression. *Exp Gerontol* 30: 631–643, 1995.
32. **Keenan TW, Franke WW, Mather IH, and Morre DJ.** Endomembrane composition and function in milk formation. In: *Lactation: A Comprehensive Treatise*, edited by Larson BL. New York: Academic, 1978, p. 404–436.
33. **Keenan TW, Morre DJ, and Haung CM.** Membranes of the mammary gland. In: *Lactation: A Comprehensive Treatise*, edited by Larson BL. New York: Academic, 1974, p. 191–233.
34. **Keeton TP, Burk SE, and Shull GE.** Alternative splicing of exons encoding the calmodulin-binding domains and C termini of plasma membrane Ca(2+)-ATPase isoforms 1, 2, 3, and 4. *J Biol Chem* 268: 2740–2748, 1993.
35. **Koszewski NJ, Reinhardt TA, Langub MC, Malluche HH, and Horst RL.** Selectivity of a C-terminal peptide antiserum for different DNA-binding states of the vitamin D receptor. *Arch Biochem Biophys* 349: 388–396, 1998.
36. **Lin P, Yao Y, Hofmeister R, Tsien RY, and Farquhar MG.** Overexpression of CALNUC (nucleobindin) increases agonist and thapsigargin releasable $Ca^{2+}$ storage in the Golgi. *J Cell Biol* 145: 279–289, 1999.
37. **Liu BF, Xu X, Fridman R, Muallem S, and Kuo TH.** Consequences of functional expression of the plasma membrane $Ca^{2+}$ pump isoform 1a. *J Biol Chem* 271: 5536–5544, 1996.
38. **Monteith GR and Roufogalis BD.** The plasma membrane calcium pump—a physiological perspective on its regulation. *Cell Calcium* 18: 459–470, 1995.
39. **Neville MC and Peaker M.** Calcium fluxes in mouse mammary tissue in vitro: intracellular and extracellular calcium pools. *J Physiol (Lond)* 323: 497–517, 1982.
40. **Neville MC, Selker F, Semple K, and Watters C.** ATP-dependent calcium transport by a Golgi-enriched membrane fraction from mouse mammary gland. *J Membr Biol* 61: 97–105, 1981.
41. **Neville MC and Watters CD.** Secretion of calcium into milk: Review. *J Dairy Sci* 66: 371–380, 1983.
42. **Okorokov LA and Lehle L.** Ca(2+)-ATPases of *Saccharomyces cerevisiae*: diversity and possible role in protein sorting. *FEMS Microbiol Lett* 162: 83–91, 1998.
43. **Pezzati R, Bossi M, Podini P, Meldolesi J, and Grohovaz F.** High-resolution calcium mapping of the endoplasmic reticulum-Golgi-exocytic membrane system. Electron energy loss imaging analysis of quick frozen-freeze dried PC12 cells. *Mol Biol Cell* 8: 1501–1512, 1997.
44. **Reinhardt TA and Horst RL.** $Ca^{2+}$-ATPases and their expression in the mammary gland of pregnant and lactating rats. *Am J Physiol Cell Physiol* 276: C796–C802, 1999.
45. **Sorin A, Rosas G, and Rao R.** PMR1, a $Ca^{2+}$-ATPase in yeast Golgi, has properties distinct from sarco/endoplasmic reticulum and plasma membrane calcium pumps. *J Biol Chem* 272: 9895–9901, 1997.
46. **Stauffer TP, Guerini D, and Carafoli E.** Tissue distribution of the four gene products of the plasma membrane $Ca^{2+}$ pump. A study using specific antibodies. *J Biol Chem* 270: 12184–12190, 1995.
47. **Stauffer TP, Guerini D, Celio MR, and Carafoli E.** Immunolocalization of the plasma membrane $Ca^{2+}$ pump isoforms in the rat brain. *Brain Res* 748: 21–29, 1997.
48. **Stauffer TP, Hilfiker H, Carafoli E, and Strehler EE.** Quantitative analysis of alternative splicing options of human plasma membrane calcium pump genes. *J Biol Chem* 268: 25993–6003, 1993. [Corrigenda. *J Biol Chem* 269: December 1994, p. 32022.]
49. **Taylor RS, Jones SM, Dahl RH, Nordeen MH, and Howell KE.** Characterization of the Golgi complex cleared of proteins in transit and examination of calcium uptake activities. *Mol Biol Cell* 8: 1911–1931, 1997.
50. **Virk SS, Kirk CJ, and Shears SB.** $Ca^{2+}$ transport and $Ca^{2+}$-dependent ATP hydrolysis by Golgi vesicles from lactating rat mammary glands. *Biochem J* 226: 741–748, 1985.
51. **Wasserman RH, Chandler JS, Meyer SA, Smith CA, Brindak ME, Fullmer CS, Penniston JT, and Kumar R.** Intestinal calcium transport and calcium extrusion processes at the basolateral membrane. *J Nutr* 122: 662–671, 1992.